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Increased Antimycobacterial Immunity in Interleukin-10-Deficient Mice

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Macrophage effector functions are essential for clearing mycobacterial infections. Interleukin 10 (IL-10) negatively regulates macrophages and could be a factor inhibiting effective antimycobacterial immunity. We previously showed that transgenic mice which produce excess IL-10 from T cells are susceptible to infection, even though these mice continue to produce gamma interferon (IFN-γ) at levels similar to those in controls. Here, we extend our genetic analysis of the functions of IL-10 in antimycobacterial immunity by testing the hypothesis that IL-10-deficient (IL-10$^{-/-}$) mice should be more resistant to mycobacteria than control mice. *Mycobacterium bovis* bacillus Calmette-Guérin-infected IL-10$^{-/-}$ mice had significantly lower bacterial burdens than control mice early in the infection. Contrary to expectations, however, IL-10$^{-/-}$ mice did not have increased levels of IFN-γ, either from T cells or in the plasma, suggesting that other mechanisms are responsible for the increased resistance. However, macrophages from IL-10$^{-/-}$ mice produced increased levels of inflammatory cytokines, including IFN-γ, as well as nitric oxide and prostaglandins, which could account for increased antimycobacterial immunity. Our genetic analysis revealed that IL-10 is an inhibitor of early mycobacterial clearance. The data also suggest that IL-10 negatively regulates numerous macrophage functions as well as playing a role in down-regulating the general inflammatory response, especially in conditions where an infection must be controlled through macrophage activity.

Mycobacterial infections are controlled by the activation of infected macrophages through gamma interferon (IFN-γ) secreted by antigen-specific helper T cells. Overwhelming evidence suggests that the activation of macrophages by IFN-γ is the critical event in bacterial control (11). The mechanisms macrophages use to contain and eliminate mycobacteria include the increased expression of inducible nitric oxide synthase (iNOS), whose product, nitric oxide (NO), is toxic to intracellular pathogens (7, 8, 11, 39). Mice or humans which lack components of the IFN-γ signaling pathway, including IFN-γ, the IFN-γ receptor, receptor-activated signaling molecules, and iNOS, are highly susceptible to mycobacterial infection (10, 14, 18, 25–28, 30, 36, 37). Nevertheless, mycobacterial infections are usually chronic in nature, and thus, the activation of macrophages by IFN-γ is insufficient to produce complete immunity to the bacteria.

Interleukin 10 (IL-10) is produced mainly by T cells and is often associated with Th2 cells (32). IL-10 can also be produced by macrophages in response to stimuli, including mycobacteria and mycobacterial products such as AraLAM (lipoarabinomannan), a mycobacterial glycolipid (43, 44). IL-10 was initially found to be an inhibitor of IFN-γ production from established Th1 cell clones as well as a negative regulator of inflammation (32). Studies with IL-10-deficient (IL-10$^{-/-}$) mice support in vitro observations of IL-10 activity: T cells from IL-10-deficient mice produce more IFN-γ than do control mouse T cells (29), and IL-10-deficient mice die rapidly from *Toxoplasma* or *Trypanosoma cruzi* infection, due to systemic overproduction of inflammatory mediators such as IFN-γ, tumor necrosis factor alpha (TNF-α), and IL-12 (20, 24, 38). In contrast, IL-10$^{-/-}$ mice are more resistant to *Listeria* infection (12), possibly through the increased IFN-γ production from T cells observed in these animals.

Our previous work has suggested that IL-10 is a central regulator of the chronic state of mycobacterial infections (34). Transgenic mice which overproduce IL-10 from T cells develop a larger bacterial burden than controls but do not die or exhibit significant pathology beyond mild splenomegaly. The excess IL-10 produced by T cells does not affect IFN-γ production; in fact, the mice have a robust Th1 response (34). These results led us to propose that the excess IL-10 favors inhibition of macrophage activation, even though IFN-γ is readily detected. These results are supported by in vitro studies which show that administration of IL-10 to mycobacterium-infected macrophages inhibits bacterial killing initiated by IFN-γ (17).

Given strong evidence that IL-10 is a negative regulator of macrophage function, we hypothesized that IL-10$^{-/-}$ mice should clear the infection faster than control mice, indicating a central role for this cytokine in the set point between latency and clearance of mycobacteria.

MATERIALS AND METHODS

Mice and infections. IL-10$^{-/-}$ mice (29), backcrossed approximately eight generations onto the C57BL/6 background, were originally obtained from the Jackson Laboratories (Bar Harbor, Maine) and bred in the conventional housing facility at the Whitehead Institute or St. Jude Children's Research Hospital. Age- and sex-matched C57BL/6 mice or littermates from IL-10$^{+/-}$ crosses were used as controls. Mice were housed and bred under conventional conditions until the time of infection, when they were transferred into a biohazard level 2 facility. Mice (8 to 12 weeks of age in all experiments) were infected intraperitoneally (i.p.) in all experiments with a single dose of ~10^5 to 10^7 CFU of *Mycobacterium bovis* bacillus Calmette-Guérin Pasteur strain, depending on the experiment. Bacterial numbers injected in each experiment were quantitated by plating dilutions onto MH9 plates and scoring colonies 21 days later. For the experiment for which results are shown in Fig. 6, 5×10^4 CFU were injected i.p. to reduce the toxic effects of larger doses of BCG in the IFN-γ$^{-/-}$ background. Under these conditions, a different time schedule is required to see a difference between control mice and IL-10$^{-/-}$ mice.

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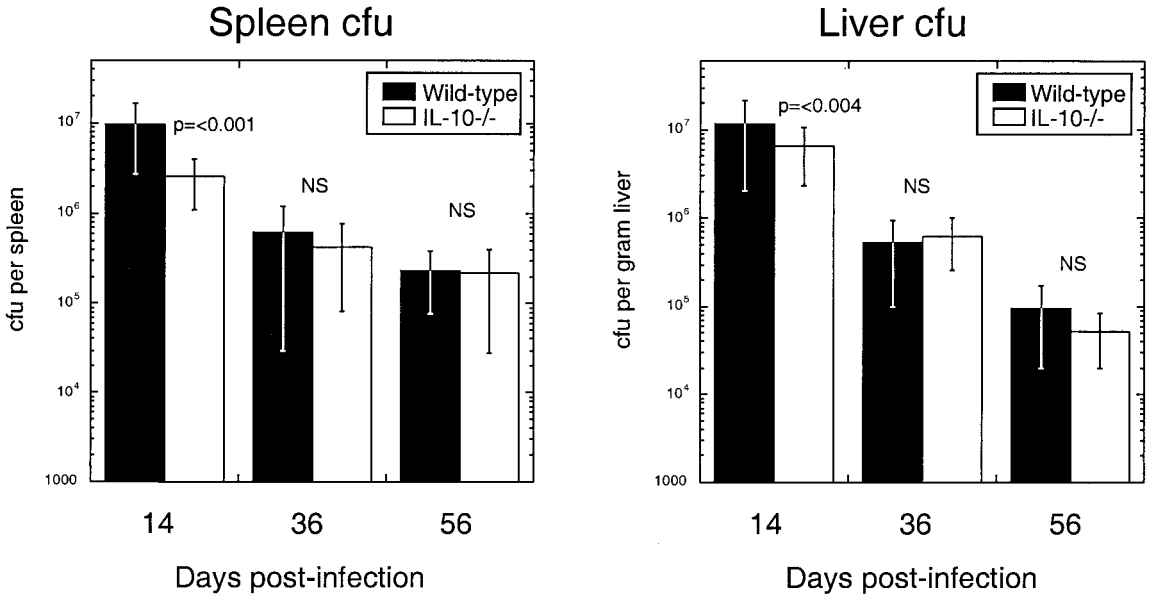

FIG. 1. IL-$10^{-/-}$ mice have increased early resistance to BCG infection. Mice (n = 8 per group) were infected with 10^7 CFU of BCG. Mice were sacrificed at the times shown on the abscissa, and organ homogenates were plated onto MH9 plates. Colony numbers were counted 25 days later and analyzed by the Mann-Whitney rank sum test. Data are presented as median values with the error bars spanning the 75th to 25th percentiles. NS, not statistically significant. Data are representative of results of three similar experiments.

Bacteria were grown and prepared for injection as described previously (33, 35). The disrupted allele of the IL-10 gene was detected when necessary by PCR of tail DNA by using the following primers: PM133, TAGGCGAATGTTCTT CC; PM134, CCTGCGTGCAATCCATCTTG; and PM189, GTACTGGCCCC TGCTGATCCTC. The combination of PM133 and PM134 identifies the disrupted allele (~1.3 kbp), while the combination of PM133 and PM189 identifies the wild-type allele (~1.0 kbp). IL-$10^{-/-}$ IFN-$\gamma^{-/-}$ doubly deficient mice were constructed by intercrossing IL-$10^{-/-}$ and IFN-$\gamma^{-/-}$ mice (both on the C57BL/6 background); this will be described in detail in a future publication.

Mycobacterial counts, histological analysis, and hematological analysis. Mycobacterial loads in the spleen and liver were quantitated by plating dilutions of spleen or liver homogenates onto MH9 plates as described previously (33). Organs were homogenized in a final volume of 10 ml and serially diluted in phosphate-buffered saline (PBS)–0.05% Tween-80. Homogenates were plated onto MH9 plates, and colony numbers were scored 21 to 28 days later. For histological analysis, livers and spleens were fixed in phosphate-buffered formalin and embedded, and sections were stained with hematoxylin and eosin stain or with the Ziehl-Neelsen stain for acid-fast bacteria. Blood smears and cytospin analysis of spleen cells were performed as described previously (36). Differential counts (counting of 100 to 300 cells) were measured on blood smears or spleen preparations of individual mice. Hematocrits were measured from blood collected at the terminal bleed into heparinized hematocrit tubes and are reported as percentages. Cytokines present in the plasma were measured by enzyme-linked immunosorbent assay (ELISA) by using reagents as previously described (33).

Antigen-specific T-cell responses. Splenocytes from infected mice were isolated on Ficoll gradients and stimulated with purified protein derivative (a mycobacterial antigen mixture) or concanavalin A (ConA) as described previously (33). Cytokines produced in response to PPD stimulation were measured by ELISA.

Macrophage isolation and stimulation. Peritoneum-derived macrophages (PDMs) were isolated from the peritoneal cavities of mice which had been injected with 1 to 2 ml of 3% Brewer's thioglycollate medium. Cells were flushed from the cavity 5 days after injection by using ~8 ml of RPMI administered through a 21-gauge needle, washed with complete media, counted, and plated at 2×10^6 cells per ml in RPMI with 10% fetal bovine serum. Cells were allowed to rest overnight and then stimulated with lipopolysaccharide (LPS) or LPS and IFN-γ at the concentrations described in the legends for Fig. 4 and 5. LPS or cytokines were made up in complete RPMI to the appropriate concentrations and added directly to cells.

NO and prostaglandin measurements. Nitrate levels in cell supernatants were measured by using the Griess reagent (45). Prostaglandin E_2 levels were measured by a competitive ELISA (Biomol, Plymouth Meeting, Pa.).

Immunoblotting. Cell lysates were made by using RIPA buffer (1% Nonidet P-40, 0.5% sodium deoxycholate, 0.1% sodium dodecyl sulfate [SDS] made up in PBS). Just prior to use, sodium orthovanadate (1 mM), phenylmethylsulfonyl fluoride (100 μg/ml), and protease inhibitor cocktail (9) were added. Cells were washed three times with ice-cold PBS. Two hundred microliters of RIPA buffer was added per well of a six-well plate, and the cells were scraped from the plate with the end of a 1-ml syringe plunger. Lysates were centrifuged in a minicentrifuge for 5 min at 4°C. Each lysate was subsequently aliquotted and stored frozen at −70°C. Five to ten microliters of each sample was analyzed by gel electrophoresis. Samples for electrophoresis were boiled in SDS sample buffer and loaded onto 4 to 15% gradient SDS–polyacrylamide gel electrophoresis (PAGE) gels (Bio-Rad, Richmond, Calif.). Western blotting was performed as described previously (9), and chemiluminescence (Amersham, Arlington Heights, Ill.) was used as the final readout. Rabbit polyclonal antibodies to IRF-1 were from Santa Cruz Biotechnology (Santa Cruz, Calif.). A polyclonal antibody to iNOS was from Biomol. Monoclonal antibodies to COX-2 and Grb2 were purchased from Transduction Laboratories (Lexington, Ky.).

Statistical methods. Data were analyzed by the Mann-Whitney rank sum test for data that are not expected to fall within a normal distribution. Data (see Fig. 1) are presented as medians with the error bars spanning the 75th to 25th percentiles.

RESULTS

IL-10-deficient mice have increased resistance to mycobacterial infection. IL-$10^{-/-}$ mice or control C57BL/6 mice were infected with different amounts of BCG (ranging from 10^5 to 10^7 CFU depending on the experiment), and the course of infection was followed over time. IL-$10^{-/-}$ mice had statistically significantly lower numbers of bacteria in the spleen and liver, but this effect was evident only in the first 2 weeks of infection (Fig. 1). Following the early part of the infection, both IL-$10^{-/-}$ mice and control mice had similar bacterial burdens. A robust granulomatous response in the liver and spleen was observed in both IL-$10^{-/-}$ mice and control mice. However, compared to control mice, some IL-$10^{-/-}$ mice had fewer acid-fast bacteria in the lesions and fewer areas of bacterium-infested necrosis (data not shown). These results suggest that the absence of IL-10 favors clearance of bacteria, but only during the initial phase of the infection.

In IL-10-deficient mice, T-cell response to mycobacteria is similar to that in control animals. Since previous studies have shown that IFN-γ production is enhanced in the absence of IL-10 (1, 12, 29), it was of importance to test the T-cell re-

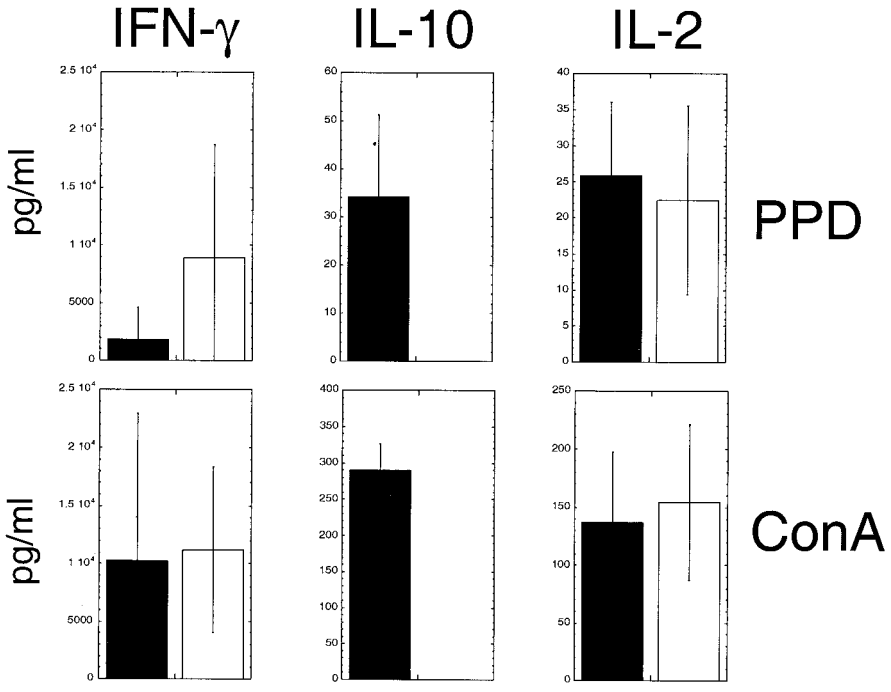

FIG. 2. IFN-γ production from spleen cells. IL-10$^{-/-}$ mice (open bars) or wild-type mice (closed bars) were infected with BCG. Four weeks later, splenocytes were isolated and stimulated with mycobacterial antigens (PPD, upper graphs) or ConA (lower graphs). Cytokines present in the culture supernatants were measured at 24 h (IL-2) or 72 h (other cytokines) poststimulation. Results are the means (error bars are the standard deviations) of results from duplicate cultures of splenocytes from six individual mice per group. In the absence of stimuli (i.e., splenocytes plated in media alone) the cells did not produce the cytokines assayed above background levels. Results are representative of three individual experiments performed to test T-cell responses to PPD.

sponse to mycobacteria in IL-10$^{-/-}$ mice. IFN-γ production from PPD- or ConA-stimulated T cells from IL-10$^{-/-}$ mice was similar to that from these cells from control mice (Fig. 2). In addition, we found no evidence of any differences in plasma IFN-γ levels during the infection (see Fig. 3A). Statistical analysis of multiple experiments also revealed no significant differences between the groups of mice. Other cytokines produced in response to these stimuli were essentially identical to those produced by wild-type T cells (data not shown).

Hematological parameters in IL-10-deficient mice. When infected with *Toxoplasma* or *T. cruzi*, IL-10$^{-/-}$ mice develop an uncontrolled pathological outpouring of inflammatory mediators, such as TNF-α, IL-12, IFN-γ, and IL-1, which is considered to be the major contributor to the early death of these mice (19). We tested if this was also the case in mycobacterial infection. Levels of IL-6, IL-12, IFN-γ, and TNF-α in plasma were measured at various times after infection (Fig. 3A). Plasma IFN-γ levels in both wild-type mice and IL-10$^{-/-}$ mice peaked around 1 month postinfection and declined thereafter, consistent with previous observations (36). Some IL-10$^{-/-}$ mice had higher levels of IL-12 and IL-6, but cytokine levels were generally similar to those for controls. TNF-α was not detected in the plasma from any mice (data not shown). These results suggest that, in contrast to *Toxoplasma* or *T. cruzi* infection, IL-10$^{-/-}$ mice infected with mycobacteria do not overproduce proinflammatory cytokines. We measured the total leukocyte counts and leukocyte differential counts in all experiments to determine if there was a correlation between increased resistance and granulocyte production. IL-10$^{-/-}$ mice had slightly higher levels of leukocytes at all time points after infection (Fig. 3B). IL-10$^{-/-}$ mice also develop a chronic anemia of unknown etiology (29). The hematocrit of infected mice remained steady over the course of infection (Fig. 3C).

Macrophages from IL-10$^{-/-}$ mice produce increased levels of inflammatory mediators. Because IL-10 inhibits inflammatory mediator production from macrophages, it is reasonable to expect that the absence of IL-10 should favor increased production of molecules such as NO, prostaglandins, and cytokines, such as TNF-α. Accordingly, we tested this by isolating inflammatory macrophages from the peritoneal cavity (PDMs) and stimulating them with agonists such as LPS and IFN-γ. If IL-10 is an essential suppressor of macrophage functions, then the cultures from IL-10$^{-/-}$ mice should produce increased levels of inflammatory mediators since IL-10 is normally produced from macrophages (32) and would act in a paracrine or autocrine fashion within the cultures.

PDMs from IL-10$^{-/-}$ mice produced increased levels of iNOS and COX-2 compared to PDMs from control mice (Fig. 4). The increased levels of these enzymes correlated with increased production of NO and prostaglandin E_2. The increased expression was most evident when cells were stimulated with LPS alone. Importantly, addition of IL-10 to PDMs from IL-10$^{-/-}$ mice restored the inhibition normally observed (particularly with COX-2 expression) when these cells were treated with IL-10 and then exposed to either LPS or LPS and IFN-γ. These results show that PDMs from IL-10$^{-/-}$ mice produce increased levels of iNOS and COX-2 but can respond normally to IL-10 added exogenously.

A similar series of experiments was performed to test if cytokine production by PDMs from IL-10$^{-/-}$ mice is also increased. We expected that this would be the case given the in vivo results obtained for *Toxoplasma*- or *T. cruzi*-infected IL-10$^{-/-}$ mice. PDMs from IL-10$^{-/-}$ mice produced significantly more TNF-α, IFN-γ (Fig. 5), and IL-12 (data not shown) than PDMs from control mice when the cells were stimulated with increasing doses of LPS. Levels of IL-6 produced in response

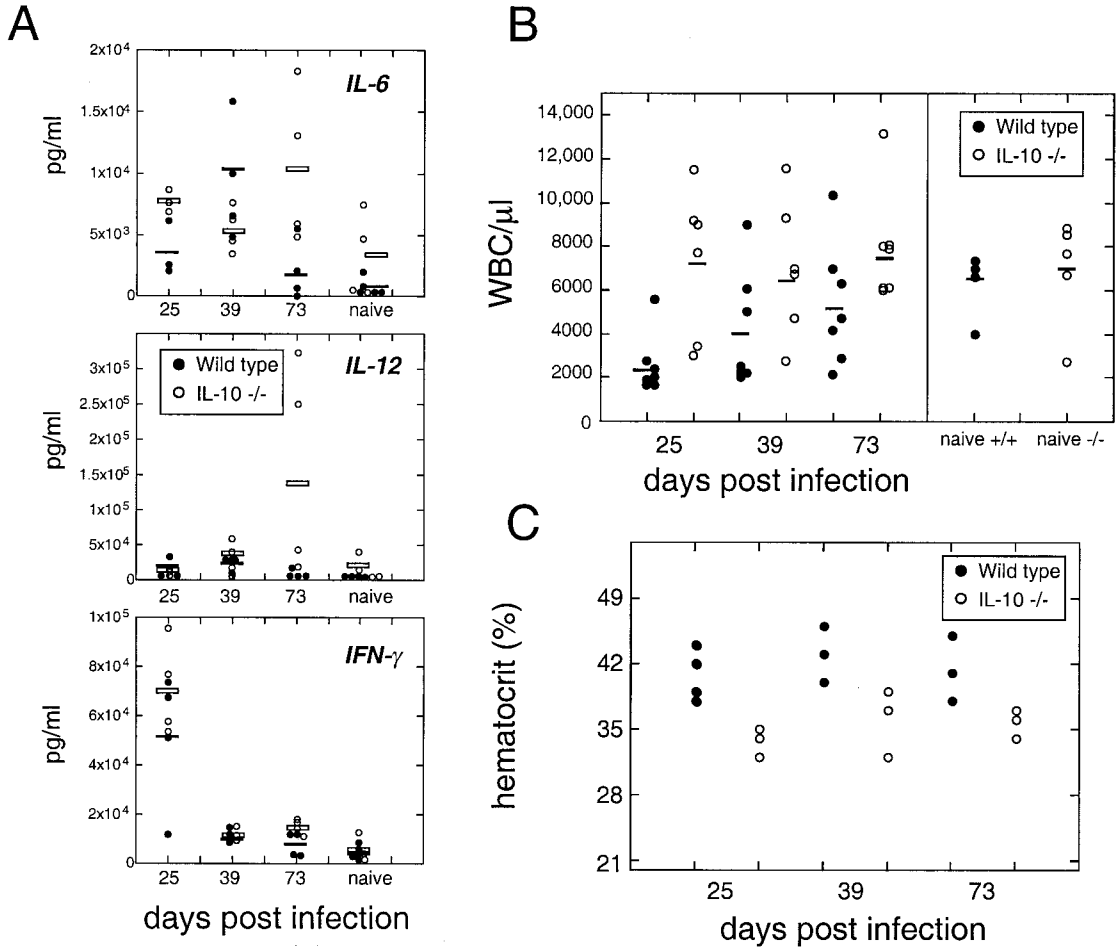

FIG. 3. Plasma cytokine levels and blood leukocyte (WBC) levels in infected mice. (A) Plasma cytokine levels. Levels of IL-6, IL-12, and IFN-γ were measured by cytokine-specific ELISA at various times after infection (in days). Results are reported in picograms per milliliter for samples from individual mice (IL-$10^{-/-}$ mice [open circles] or wild-type mice [closed circles]) from a single experiment of three performed with similar results. Plasma cytokine levels in uninfected mice (naive) are shown on the right for each cytokine. Mean values are indicated on the figure by closed (wild-type mice) or open (IL-$10^{-/-}$ mice) bars. (B) WBC levels in the blood of infected mice. WBC numbers were measured at various times after infection. Results are reported for individual mice (IL-$10^{-/-}$ mice [open circles] or wild-type mice [closed circles]) from a single experiment of three performed. WBC levels in uninfected mice (naive) are shown on the right. The average number of leukocytes is shown with a bar. (C) Hematocrits of infected mice. Hematocrits were measured for some of the mice for which data are shown in panel B.

to LPS were the same irrespective of the genotype of the cells (Fig. 5). Cytokine production was inhibited when IL-10 was added to the cultures, indicating that PDMs from IL-$10^{-/-}$ mice retain the capacity to respond to IL-10 (Fig. 5). These results show that IL-10 has a nonredundant role as a negative regulator of cytokine production from inflammatory macrophages.

Mice lacking both IL-10 and IFN-γ have a massive pathological granulocytic response. IFN-$\gamma^{-/-}$ mice develop a chronic, pathological granulocytosis when infected with mycobacteria (36). We have hypothesized that this is a response initiated when macrophage activation has failed and bacterial growth proceeds unabated. We wanted to test if IL-10 plays a role in partly suppressing the inflammatory response in infected IFN-$\gamma^{-/-}$ mice. Doubly deficient, IFN-$\gamma^{-/-}$ IL-$10^{-/-}$, mice were infected with mycobacteria and compared with IFN-$\gamma^{-/-}$, IL-$10^{-/-}$, and wild-type animals. Surprisingly, the doubly deficient mice rapidly developed a wasting syndrome and were visibly ill 1 week after infection. The mice had a massive granulocytosis that was stronger than that in IFN-$\gamma^{-/-}$ mice as well as high levels of IL-12 in plasma (Fig. 6). These results suggest that in infected IFN-$\gamma^{-/-}$ mice, IL-10 plays a role in partially suppressing an inflammatory response. As expected, the T-cell response from IFN-$\gamma^{-/-}$ IL-$10^{-/-}$ mice was Th2 oriented (data not shown).

DISCUSSION

The present study further defines the role of IL-10 in the immune response to mycobacteria by demonstrating that the absence of IL-10 favors increased resistance to these organisms but only in the early phase of the infection. The resistance is likely to be mediated through increased macrophage activity, such as elevated inflammatory mediator production. Previously, we demonstrated that transgenic mice which secrete increased levels of IL-10 from T cells are more susceptible to mycobacterial infection, despite the fact that IFN-γ levels were similar to those for wild-type mice (34). In that case, the balance of more IL-10 relative to IFN-γ favors bacterial growth, presumably by decreasing macrophage ability to eradicate the bacteria. Below, we discuss antimycobacterial immunity in mice which have altered ratios of IL-10 to IFN-γ and the implications of these observations for the mechanisms of action of these two cytokines on macrophages.

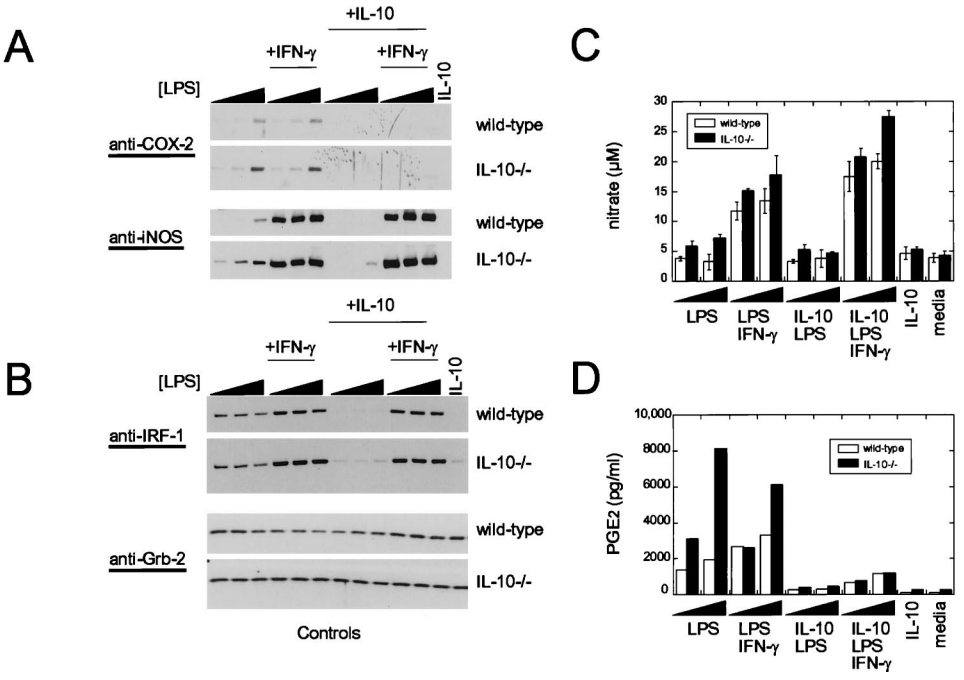

FIG. 4. Increased levels of inflammatory mediators produced from IL-10$^{-/-}$ macrophages. (A) Increased levels of COX-2 and iNOS proteins in macrophages from IL-10$^{-/-}$ mice. Peritoneal macrophages were harvested from C57BL/6 (wild-type) or IL-10$^{-/-}$ mice following injection of thioglycollate medium. Cells were stimulated with the agents listed at the top of the figure (LPS, 1, 10 or 100 ng/ml; IL-10, 10 ng/ml; and IFN-γ, 2 ng/ml) and assayed 16 h later by immunoblotting for COX-2 or iNOS proteins. Note that macrophages from IL-10$^{-/-}$ mice have slightly increased levels of both proteins. COX-2 levels are increased in these macrophages, particularly with LPS stimulation alone. (B) Loading controls for the experiment for which data are shown in panel A. The same immunoblots were reprobed with antibodies to IRF-1, as a control for the induction of IFN-γ-regulated genes, or to Grb-2, as a loading control for all samples. (C) Production of nitrate (as a marker for NO production). Supernatants from the experiment for which data are shown in panels A and B were assayed for nitrate production by using the Griess reagent. Data are from quadruplicate samples. Data from similar experiments showed that the differences in nitrate production from stimulated macrophages from IL-10$^{-/-}$ mice and control mice were statistically different ($P < 0.001$) when tested by using the t test. (D) Production of prostaglandin E_2 (PGE2) (as a marker for COX-2 activity) from an experiment similar to that shown in panels A and B. Data are from triplicate samples. LPS concentrations for panels C and D were 10 and 100 ng/ml, respectively. Data are representative of results of many similar experiments performed to determine if macrophages from IL-10−/− mice have increased inflammatory-response mediator production.

Based upon previous knowledge of the actions of IL-10 on macrophages, it was expected that IL-10$^{-/-}$ mice would have increased resistance to mycobacterial infection, due to a higher output of IFN-γ, which would lead to increased macrophage antibacterial activity. Our data showing increased early resistance partially supports this hypothesis, but we suggest that a more likely scenario is that IFN-γ can activate macrophages in the absence of IL-10, independent of increases in IFN-γ levels. IL-10$^{-/-}$ mice infected by an aerosol challenge with a different strain of BCG did not show differences in bacterial numbers or increased IFN-γ production (16). The discrepancy with the present study is possibly related to the site of infection. There may be specific differences in the role of IL-10 in the lung as the initial site of infection compared to those in the spleen and liver. Given that the mycobacteria begin the infection process and reside predominantly within the lung, these questions warrant further investigation.

Using a pharmacological approach, two other groups have shown that systemic neutralization of IL-10 augmented resistance to *Mycobacterium avium* infection (2, 13). These data agree with our genetic approach presented here. Furthermore, the fact that anti-IL-10 antibodies can decrease bacterial loads in mycobacterial infection may suggest new opportunities for therapeutic interventions in tuberculosis. The neutralization studies did not, however, determine any mechanistic basis for the increased resistance (2, 13). In addition, the data must be interpreted in the light of the more general role of IL-10 in modulating the inflammatory response. Systemic neutralization of IL-10 may cause adverse effects on pathways where inflammation is essential for efficient control of pathogens.

Toxoplasma infection of IL-10$^{-/-}$ mice produced several dramatic results (20, 38). The mice died rapidly, but without a significant increase in parasite burden. The cause of death was suggested to be overproduction of proinflammatory cytokines from CD4^{+} T cells (20, 38). The present study shows that mycobacterial infection of IL-10$^{-/-}$ mice does not engender a massive systemic inflammatory response. This may be a consequence of the more limited host cell range of mycobacteria (macrophages) compared to that of *Toxoplasma* (potentially all nucleated cells). The function of IL-10 in suppressing an inflammatory response in a mycobacterial infection was revealed when IFN-γ$^{-/-}$ IL-10$^{-/-}$ mice were infected. IFN-γ$^{-/-}$ mice develop a pathologic granulocytosis accompanied by complete remodeling of the hematopoietic system to favor granulocyte production (36). When IL-10 was absent in the IFN-γ-deficient background, the inflammatory response was increased to the point at which animals were dying 2 weeks after infection, concomitant with detection of large amounts of IL-12 in their plasma, a large bacterial burden, and leukocytosis. The simplest interpretation of these results is that IL-10 suppresses inflammatory mediator production in mycobacterium-infected IFN-γ$^{-/-}$ mice. This observation is similar to that observed for *Toxoplasma* infection of IL-10$^{-/-}$ mice and further confirms the essential role of IL-10 as a general negative regulator of inflammation. Finally, the role of IL-10 in systemic regulation of inflammatory responses was independently shown by treat-

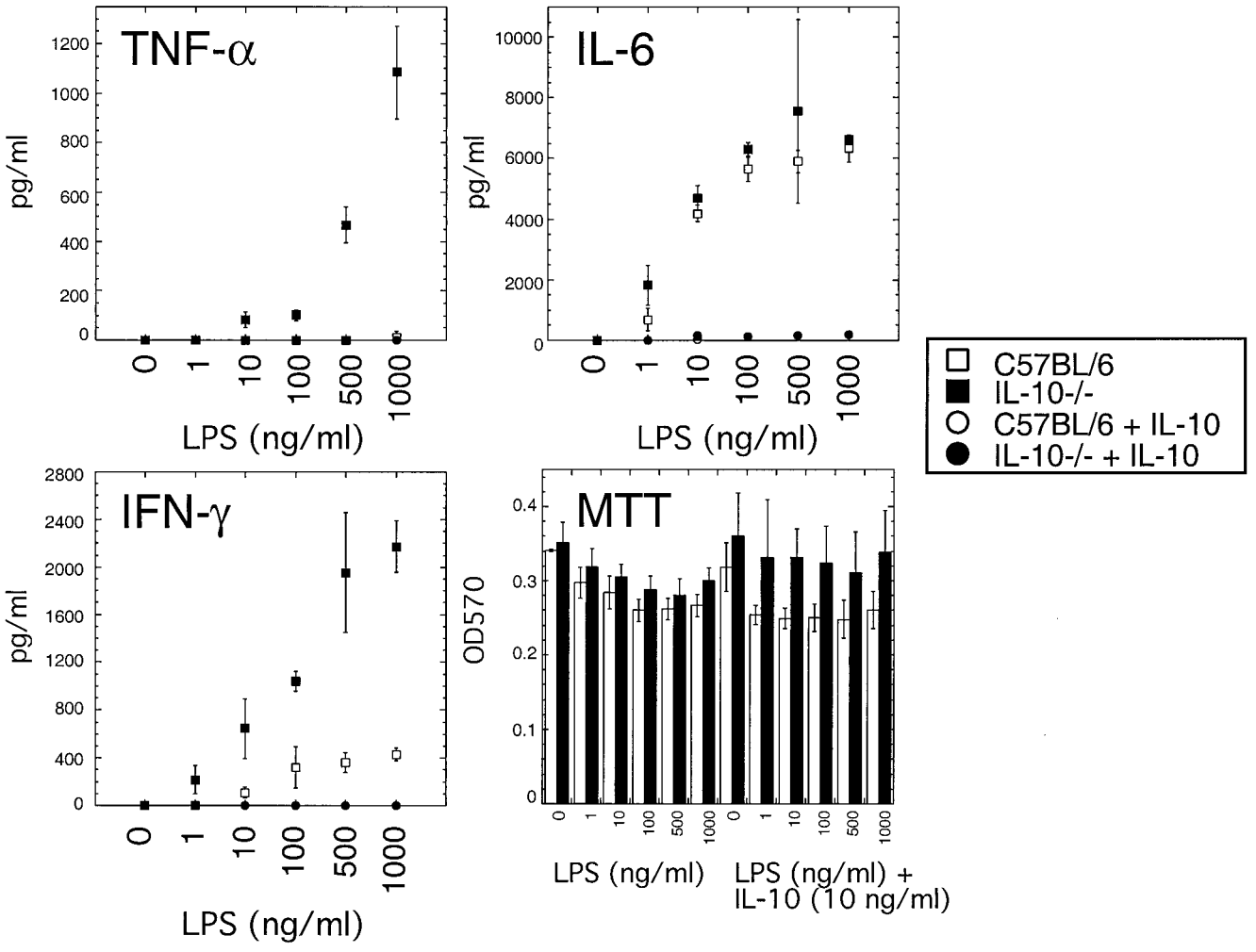

FIG. 5. Increased levels of inflammatory cytokines produced from IL-$10^{-/-}$ macrophages. Peritoneal macrophages were isolated from C57BL/6 mice (open symbols) or IL-$10^{-/-}$ mice (closed symbols). Adherent cells were rested in vitro for 24 h prior to stimulation with increasing doses of LPS ranging from 0 to 1,000 ng/ml with or without the addition of 10 ng of IL-10/ml. Culture supernatants were assayed 16 h after stimulation by cytokine-specific ELISA. Cell viability at the end point of the assay was measured by the addition of MTT (5 μg/ml) for 4 h. Cells were washed with PBS, and the reduced dye was dissolved with acid isopropanol. Aliquots of each sample were read in an ELISA reader at OD_{570}. Data are means with standard deviations from quadruplicate samples of one experiment of two performed with similar results. MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide.

ment of IL-$10^{-/-}$ mice with staphylococcal enterotoxin, which also evoked a massive outpouring of inflammatory cytokines as well as increased plasma NO levels (21).

Our analysis of macrophages isolated from IL-$10^{-/-}$ mice allowed us to speculate on the mechanisms of increased early resistance to BCG infection. Macrophages from IL-$10^{-/-}$ mice produced slightly elevated levels of iNOS and NO and much higher levels of prostaglandin E_2, which was used as a marker of prostanoid production, than those from control mice. COX-2 protein levels were also increased, accounting for the elevated prostanoid production. In addition, inflammatory cytokine production, including IFN-γ production, was elevated when IL-$10^{-/-}$ macrophages were stimulated with LPS. Thus, our study confirms previous work demonstrating that IL-10 is a powerful inhibitor of NO, TNF-α, and COX-2 production (4, 5, 31, 40, 41) by demonstrating that IL-10 is an essential inhibitor of pathways that produce a variety of inflammatory-response mediators.

A remaining question concerns the reasons why increased resistance was observed only early in the infection. If IL-10 were the sole negative mediator of antimycobacterial immunity, then we would expect to find that the bacterial burden diminished rapidly compared to that in controls. In contrast, we observed that after ~30 days of infection, bacterial numbers were similar in IL-$10^{-/-}$ mice and controls. This suggests that other factors may play a role in inhibiting clearance of mycobacteria, including IL-4 and transforming growth factor β, which have previously been shown to have negative effects on macrophage activity (3, 6, 46–48). There are also other possibilities which have not been explored to date, including the possibility that IL-10 plays a role in granuloma formation or in the specific inhibition of a cell type required for migration to granulomas. Given that we did not observe any differences in granuloma number or appearance (other than the observation that wild-type animals occasionally have more acid-fast bacteria in necrotic granulomas), the former possibility seems unlikely. More experiments are required to test if IL-10 affects, for example, a specific T-cell type or macrophages that must form granulomas early in the infection process to control bacterial growth.

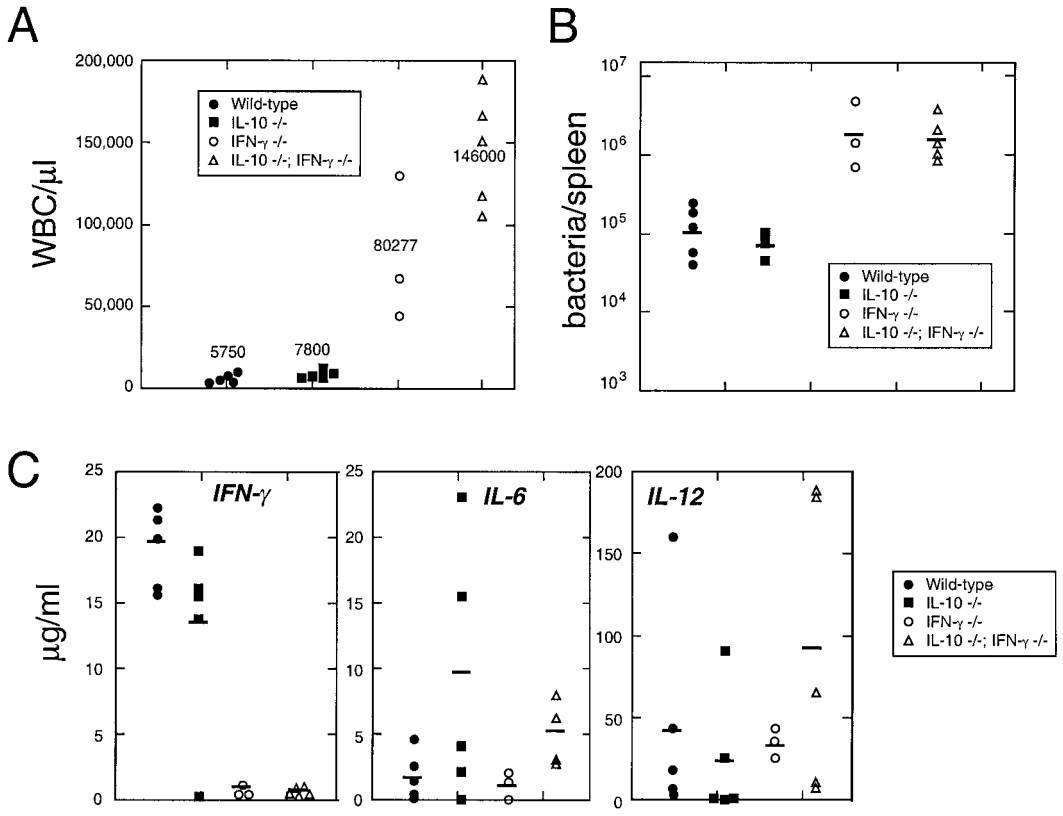

FIG. 6. Mycobacterial infection of mice lacking both IL-10 and IFN-γ. Mice of each genotype were infected with 5×10^4 CFU i.p. and sacrificed 2 weeks later. All results are reported in terms of individual mice from one experiment of two performed with similar results. (A) Leukocyte (WBC) numbers from infected mice. Mean values are indicated in the panel. (B) Bacterial numbers in the spleens of infected mice. Mean values are indicated with bars. (C) Levels of IFN-γ, IL-6, and IL-12 in the plasma of infected mice. Mean values are indicated with bars.

Our data allow us to consolidate the current views of the roles of IFN-γ and IL-10 in antimycobacterial immunity. Fig. 7 shows a diagrammatic representation of the mouse strains we have studied using the BCG infection system. Previous work had identified IFN-γ$^{-/-}$ mice as being highly susceptible to infection (10, 11, 18). These mice have a Th2-cell response (because IFN-γ plays a nonredundant role in the mounting of a Th1 response) and an uncontrolled, pathological granulocy-

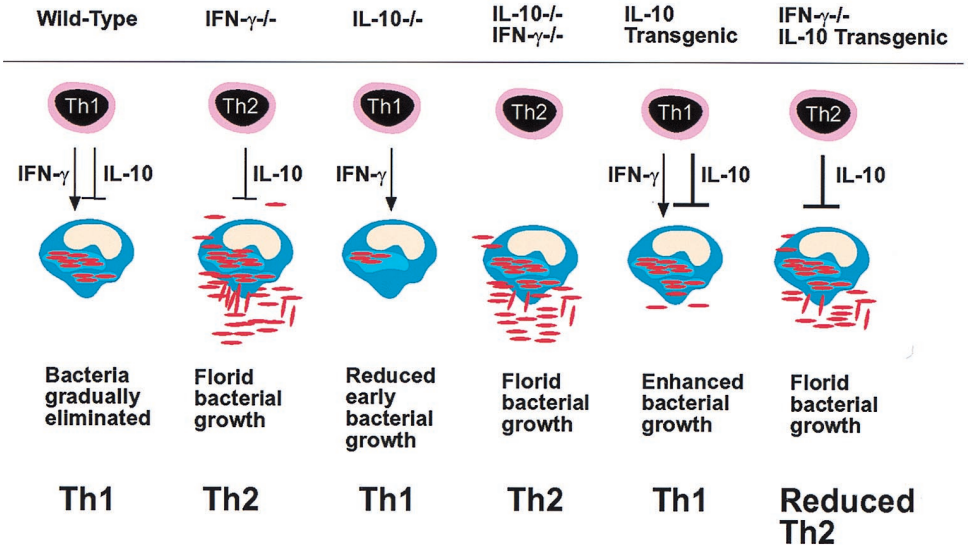

FIG. 7. Summary of the roles of IL-10 and IFN-γ in antimycobacterial immunity. The strain of mice is shown at the top along with a schematic representation of the T-cell–infected-macrophage interaction. The type of T cell responding to mycobacterial infection is shown on the bottom. Note that in the absence of IFN-γ, T helper development defaults to the Th2 phenotype.

tosis, which is likely to be a secondary attempt to control bacterial growth (36). In contrast to the role of IFN-γ, IL-10 overexpression tips the balance in favor of bacterial growth (34) while loss of IL-10 causes increased resistance in the early part of the infection (the period examined in this study). We constructed two other strains to confirm the roles of these two cytokines in antimycobacterial immunity. In the first, the IL-10 transgenes (34) were crossed into the IFN-$\gamma^{-/-}$ background (36a). These mice were also highly susceptible to BCG infection. The only obvious difference between these mice and IFN-$\gamma^{-/-}$ mice was a diminution in the Th2 response, presumably caused by excess IL-10. There was, however, no effect on limiting bacterial growth or the pathologic granulocytosis. We also constructed mice which lack both IFN-γ and IL-10. In this background, we anticipated that bacterial growth would continue unabated, which was observed. T cells from these mice produce abundant IL-3, IL-4, and IL-5, indicative of a robust Th2 response (data not shown). The mice died very rapidly from mycobacterial infection, which we believe to have been caused by the granulocytic response which went on unchecked in the absence of IL-10. This result correlated well with previous observations on the infection of IL-$10^{-/-}$ mice with *Toxoplasma* as discussed above.

IFN-γ and IL-10 have a multitude of effects on the immune system that have been revealed by infecting genetically altered mice with a variety of pathogens. IFN-γ is involved in the establishment and propagation of a Th1 response which itself is responsible for antigen-specific T-cell IFN-γ production. IFN-γ is also essential for the activation of macrophages to kill intracellular pathogens. On the other hand, IL-10 is a major suppressor of the inflammatory response to pathogens, a suppressor of macrophage function, and a regulator of IFN-γ production from T cells. Surprisingly, IL-10 is closely related to IFN-γ both structurally and in signaling mechanisms (15, 22, 23, 42, 49). How IL-10 and IFN-γ coordinately regulate macrophage function, inflammation, and T-cell activity is currently a topic of great interest in understanding immune responses.

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